J. Biochem. **137**, 331–338 (2005)
DOI: 10.1093/jb/mvi036

Involvement of Calpain in Osteoclastic Bone Resorption

Masami Hayashi[1], Yasuko Koshihara[2], Hideaki Ishibashi[3], Seizo Yamamoto[3], Satoshi Tsubuki[4], Takaomi C. Saido[4], Seiichi Kawashima[5] and Mitsushi Inomata[1,*]

[1]Biomembrane Research Group, and [2]Bone Research Group, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015; [3]Department of Orthopedic Surgery, Tokyo Metropolitan Geriatric Medical Center, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015; [4]Laboratory for Proteolytic Neuroscience, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198; and [5]Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113-8613

Received October 18, 2004; accepted December 25, 2004

There is increasing evidence that calpain contributes to the reorganization of the cytoskeleton in the integrin-mediated signaling pathway. Osteoclastic bone resorption requires cell-matrix contact, an event mediated by integrin αvβ3, and subsequent cytoskeletal reorganization to form characteristic membrane domains such as the sealing zone and ruffled border. In this study, therefore, we investigated whether calpain is involved in osteoclastic bone resorption. Membrane-permeable calpain inhibitors suppress the resorption activity of human osteoclasts, but an impermeable inhibitor does not. Upon the attachment of osteoclasts to bone, μ-calpain is translocated from the cytosolic to the cytoskeletal fraction and is autolytically activated. Both the activation of μ-calpain and the formation of actin-rings, the cytoskeletal structures essential for bone resorption, are inhibited by membrane-permeable calpain inhibitors. The activated μ-calpain in osteoclasts selectively cleaves talin, which links the matrix-recognizing integrin to the actin cytoskeleton. These findings suggest that calpain is a regulator of the bone resorption activity of osteoclasts through reorganization of the cytoskeleton related to actin-ring formation.

Key words: actin cytoskeleton, bone resorption, calpain, talin, osteoclast.

Abbreviations: calpeptin, *N*-benzyloxycarbonyl-L-leucylnorleucinal; E-64c, [L-3-*trans*-carboxyoxirane-2-carbonyl]-L-leucine(3-methylbutyl)amide; E-64d, [L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl]-L-leucine(3-methylbutyl) amide; F-actin, filamentous actin; leupeptin, acetyl-L-leucyl-L-leucyl-L-arginal; NaF, sodium fluoride; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

Calpain is an intracellular cysteine protease distributed widely in animal cells. There are two ubiquitous calpain isozymes, μ- and m-calpains, that are active at micromolar and millimolar Ca^{2+} concentrations, respectively. Both calpains consist of a μ- or m-specific 80-kDa catalytic large subunit and a common 30-kDa regulatory small subunit. Upon Ca^{2+} binding, calpains begin to autolyze and become active. In the case of μ-calpain, the 80-kDa subunit of the proenzyme is converted autolytically to a 76-kDa form *via* a 78-kDa intermediate as previously reported (*1*–*3*). Calpain activity is regulated by calcium ions and a specific endogenous inhibitor, calpastatin. Calpastatin contains four conserved calpain-inhibiting motifs (*1*). A 24–amino acid calpastatin consensus peptide has been shown to inhibit calpain specifically and not to inhibit other proteases (*4*). Although the exact physiological role of calpain is not yet established, there is increasing evidence that it plays an important role in the integrin-mediated signaling pathway. Fox *et al*. and we showed that calpain activation is triggered through the binding of an adhesive ligand to integrin αIIbβ3 and may contribute to reorganization of the cytoskeleton during platelet aggregation (*5*–*7*). In addition, accumulating evidence suggests that calpain modulates cell adhesion and motility through reorganization of the cytoskeleton during integrin-mediated signaling (*8*–*11*).

Bone resorption is performed by multinucleated cells, osteoclasts, formed by the fusion of mononuclear progenitors of the monocyte/macrophage lineage (*12*). Osteoclastic bone resorption is a multistep process that is initiated by the attachment of osteoclasts to the bone surface. It is generally believed that bone recognition by osteoclasts is controlled by an integrin receptor, αvβ3. It is also thought that this integrin transmits bone matrix–derived signals, ultimately prompting intracellular events, such as cytoskeletal reorganization, that are pivotal to bone resorption (*13*, *14*). In fact, when bone resorption is initiated, the osteoclasts become polarized and form characteristic membrane domains: the sealing zone, which is the specialized cell–extracellular matrix adhesion structure; and the ruffled border, the actual resorbing organ, which faces the bone surface called the resorption lacuna. These changes are accompanied by extensive cytoskeletal reorganization, and the actin cytoskeleton forms an attachment ring structure (podosomal ring or F-actin ring), which is essential for bone resorption, at the sealing zone (*15*, *16*). The ruffled border appears inside the sealing zone, has several characteristics of late endosomal membranes, and secretes protons and a lysosomal protease, cathepsin K, into the resorption lacuna. The secretion of protons is achieved by the action of vacuolar H^+-ATPase (V-ATPase) in the ruffled membrane. The

*To whom correspondence should be addressed. Tel: +81-3-3964-3241 (Ext. 3068), Fax: 81-3-3579-4776, E-mail: minomata@tmig.or.jp

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acidic microenvironment formed by protons first mobilizes a bone mineral, crystalline hydroxyapatite. Subsequently, the demineralized organic components of the bone are degraded by cathepsin K (*17–19*).

It has been established that cathepsin K, which is secreted from osteoclasts into the resorption lacuna, is responsible for the degradation of the collagenous bone matrix. In contrast, there is no publication describing the role of intracellular proteases in osteoclastic bone resorption. The following facts, however, suggest the involvement of calpain in osteoclastic bone resorption: (i) integrin αvβ3 plays an essential role in bone resorption (*13*, *14*); (ii) osteoclasts undergo drastic morphological and functional changes accompanied by extensive reorganization of the cytoskeleton (*15*, *16*).

In the present study, therefore, we examined whether calpain is involved in osteoclastic bone resorption as the first step in clarifing the precise role of calpain in the resorption process. We obtained interesting results that indicate the involvement of calpain in osteoclastic bone resorption. The possible role of calpain is also discussed.

MATERIALS AND METHODS

Reagents—Histopaque 1077, pronase E, PMSF (phenylmethylsulfonyl fluoride), aprotinin, and an acid phosphatase detection kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heat-inactivated horse serum and α-minimum essential medium (α-MEM) were obtained from Gibco Invitrogen (Life Technologies, Grand Island, NY, USA) and Irvine Scientific (Santa Ana, CA, USA), respectively. Rhodamine-conjugated phalloidin was a product of Molecular Probes (Eugene, OR, USA). Calpeptin was obtained from Tocris Cookson (St. Louis, MO, USA). 1α, 5-dihydroxy vitamin D_3 was generously supplied by Teijin (Tokyo, Japan). Leupeptin, E-64c, and E-64d were purchased from Peptide Institute (Osaka, Japan). A Western blotting detection system (ECL Plus) was purchased from Amersham Biosciences (Piscataway, NJ, USA). Calpastat, a cell-permeable, specific inhibitor of calpain comprising the cell-penetrating signal sequence from Kaposi's fibroblast growth factor connected to a calpain-inhibiting consensus sequence (24 amino acid) derived from calpastatin, and calpastat-ala, a mutant peptide with alanine substitutions at conserved calpastatin residues that lacks calpain-inhibitory activity, were synthesized as described previously (*20*) and purified by high performance liquid chromatography. The Live/Dead viability/cytotoxicity kit was purchased from Molecular Probes (Eugene, OR, USA). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical (Osaka, Japan).

Antibodies—A monoclonal antibody against calpain ($1D_{10}A_7$) that reacts with the large subunit of μ- and m-calpains was isolated as described before (*21*). Polyclonal antibodies specific to the pre- (80 kDa) and post-autolytic (76 kDa) forms of μ-calpain (anti–pre-μ and anti–post-μ, respectively) were raised against synthetic peptides as described previously (*22*). A monoclonal anti-intact talin (235 kDa) antibody was obtained from Novocastra Laboratories (Newcastle, UK). The antibodies specific to the calpain-generated N-terminal fragment (47 kDa) and C-terminal fragment (190 kDa) of talin were produced as described previously (*7*). An antibody against human α-actinin was produced in rabbits using a synthetic peptide corresponding to residues 878–892, DYMSFSTALYGESDL, with a cysteine residue added to the N terminus. Monoclonal anti–human vinculin and anti–human integrin αvβ3 antibodies were purchased from Sigma-Aldrich and Chemicon International (Temecula, CA, USA), respectively. Peroxidase-conjugated anti–mouse IgG and anti–rabbit IgG were obtained from Medical & Biological Laboratories (Nagoya, Japan).

Cells—Human periosteal osteoblast-like cells, SaM-1 cells, were established in culture as previously reported (*23*).

Isolation of Human Osteoclast-Like Multinucleate Cells—Bone marrow cells were obtained from the proximal region of the femur of elderly patients who gave their informed consent before artificial hip replacement surgery. Osteoclast-like multinucleate cells (MNCs) were isolated from the bone marrow cells (*24*). Briefly, the mononuclear cell–rich fraction was isolated from bone marrow cells with Histopaque 1077 and cultured in α-MEM containing 20% heat-inactivated horse serum and 10^{-8} M 1α,25-dihydroxy vitamin D_3 for 2–3 weeks at 37°C in a humidified atmosphere of 95% air/5% CO_2, and MNCs were formed. Before use, the MNCs in the culture were treated with 0.002% pronase E and 0.02% EDTA in phosphate-buffered saline [PBS (–), pH 7.4] for 5 min with agitation to remove stromal cells. Greater than 95% of the remaining cells were osteoclast-like MNCs (*25*, *26*). Isolated human osteoclast-like MNCs fulfilled the functional criteria of osteoclasts: expression of tartrate-resistant acid phosphatase (TRAP); binding of ^{125}I-labeled calcitonin; actin-ring formation; and bone resorption activity (*24*, *27*, *28*). In addition, the osteoclast-like MNCs expressed mRNAs for proteins characteristic of mature osteoclasts, such as TRAP, calcitonin receptor, carbonic anhydrase II, integrin αV-chain, and cathepsin K (*25*, *26*).

Triton X-100 Fractionation—The same number of human osteoclasts was kept in suspension or reseeded on bovine cortical bone slices in α-MEM containing 20% heat-inactivated horse serum and 10^{-8} M 1α,25-dihydroxy vitamin D_3. After 2 h, the cells were washed twice with PBS (–) containing 1 mM sodium orthovanadate, lysed for 5 min in Triton X-100 lysis buffer [10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM NaF, 23 μM leupeptin, 1 TIU/ml aprotinin and 1 mM PMSF] on ice, and scraped off the bone slices. The cell lysates were centrifuged at $15,000 \times g$ for 15 min at 4°C, and the recovered supernatants were designated as the Triton X-100–soluble fraction (TSF). The insoluble pellets were washed twice with Triton X-100 lysis buffer, and the resulting pellets were resuspended in the same buffer and designated as the Triton X-100–insoluble fraction (TIF).

Osteoclast Resorption Assay—Osteoclasts in culture were dispersed by treatment with 0.002% pronase E for 20 min. The cells were seeded onto a calcium phosphate–coated disc (Osteologic; Millenium Biologix, Ontario, Canada) (*29*) and incubated in α-MEM containing 20% horse serum and 10^{-8} M 1α,25-dihydroxy vitamin D_3 with or without protease inhibitors for 2 days. The assay was terminated by the addition of bleach to remove cells. The

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samples were examined using brightfield optics. Resorption activity was estimated from the lacuna area, which was measured by counting the mesh number inside the lacuna (*24*).

Actin-Ring Formation of Osteoclasts—Osteoclasts were cultured on a plastic dish in the presence or absence of calpain inhibitors for 2 days. The cells were fixed with 10% formalin in PBS (–) and permeabilized in 0.2% Triton X-100 for 5 min. Actin-rings in osteoclasts were visualized by rhodamine-conjugated phalloidin staining according to the method of Kanehisa *et al*. (*30*). After staining, the cells were rinsed with PBS (–) for 10 min and then mounted with PermaFluor (Pittsburgh, PA, USA).

Cell Viability—The viability of osteoclasts was tested with the Live/Dead viability/cytotoxicity kit according to the manufacturer's instructions.

Electrophoresis and Western Blot Analysis—SDS-PAGE was performed in 10% polyacrylamide gels according to the method of Laemmli (*31*). The proteins in the gels were transferred onto PVDF membranes, and the blotted proteins were incubated with the first antibody followed by incubation with peroxidase-conjugated anti–rabbit IgG (for polyclonal antibody) or anti–mouse IgG (for monoclonal antibody) as the second antibody. The antigens were visualized by enhanced chemiluminescence (ECL Plus). The amounts of antigens were determined by densitometric scanning on a Shimadzu CS-9000 (Kyoto, Japan).

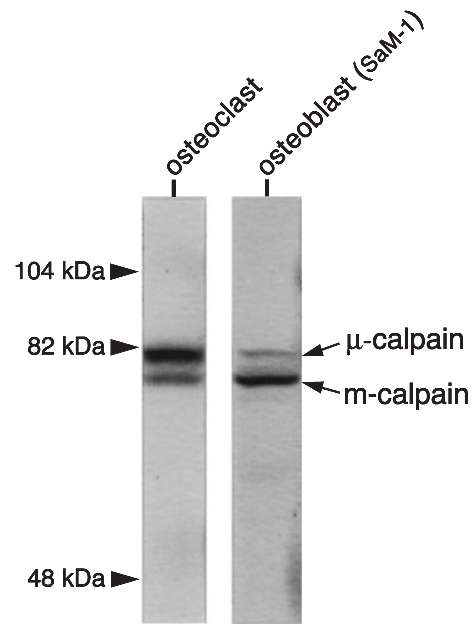

Fig. 1. **Distribution of μ- and m-calpains in human osteoclasts.** Lysates prepared from human osteoclasts or human osteoblast-like (SaM-1) cells were electrophoresed in a 10% SDS–polyacrylamide gel and electrotransferred to a PVDF membrane. Calpain antigens were detected by Western blotting using a monoclonal anti-calpain antibody ($1D_{10}$ A_7) that reacts with the large subunit of both types of calpain.

RESULTS

Distribution of μ- and m-Calpains in Human Osteoclasts—It is well known that μ- and m-calpains are distributed widely in various types of cells, although their ratio varies from cell to cell. However, there is no report describing the distribution of calpains in osteoclasts. We, therefore, examined the distribution of calpains in human osteoclasts by Western blotting. As shown in Fig. 1, human osteoclasts and osteoblast-like (SaM-1) cells contain both μ- and m-calpains; however, osteoclasts contain predominantly μ-calpain, while osteoblast-like (SaM-1) cells contain mainly m-calpain. The μ-/m-calpain ratio in osteoclasts resembles that in platelets (*32*). Based on this result, only the behavior of μ-calpain was followed in the subsequent experiments.

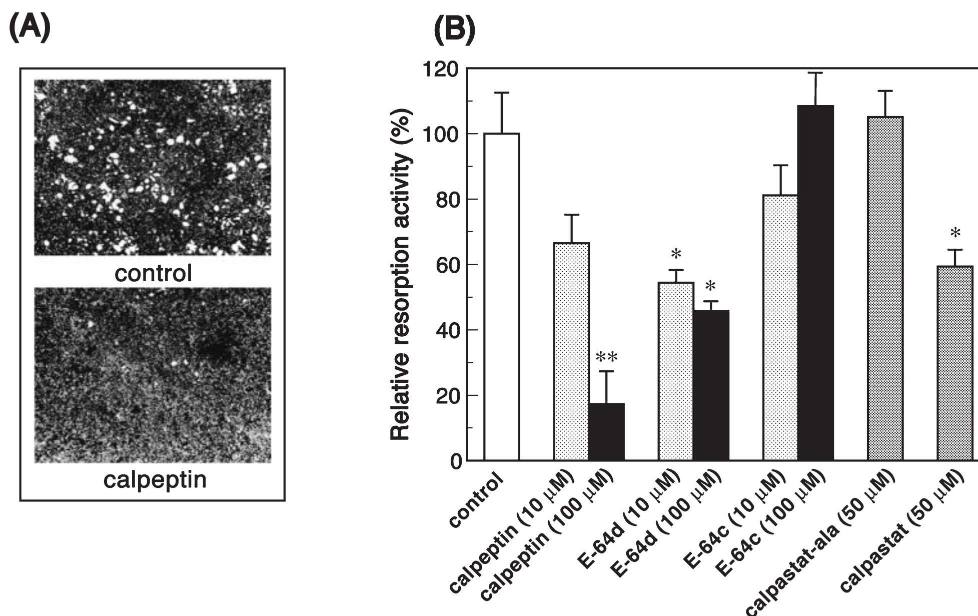

Fig. 2. **Inhibition of the resorption activity of osteoclasts by membrane-permeable calpain inhibitors.** Human osteoclasts were cultured on hydroxyapatite-coated discs (osteological discs) in the presence or absence of calpain inhibitors for 2 days. (A) Typical examples of the formation of resorption lacunae and its inhibition by treatment with 100 μM calpeptin, a membrane-permeable calpain inhibitor. White spots show resorption lacunae formed on an osteological disc by osteoclasts. (B) Effect of various calpain inhibitors on the resorption activity of osteoclasts. Resorption activity was determined by measuring the area of the resorption lacunae. Each bar represents the mean ± SE of three individual experiments. Significantly different from control at $^*p < 0.05$, $^{**}p < 0.01$.

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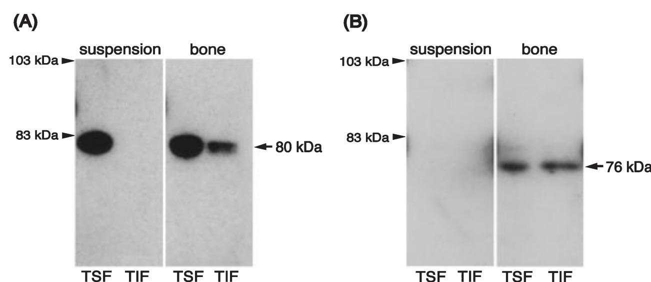


Fig. 3. **Translocation and autolytic activation of μ-calpain in osteoclasts upon attachment to bone.** The same number of osteoclasts were kept in suspension or allowed to attach to bone for 2 h. The cells were then lysed in 0.5% Triton X-100–containing lysis buffer, fractionated into the Triton X-100–soluble (TSF) cytosolic and Triton X-100–insoluble (TIF) cytoskeletal fractions, and used for Western blotting. (A) Immunostained with anti–pre-μ-calpain (80 kDa) antibody. (B) Immunostained with anti–post-μ-calpain (76 kDa) antibody.

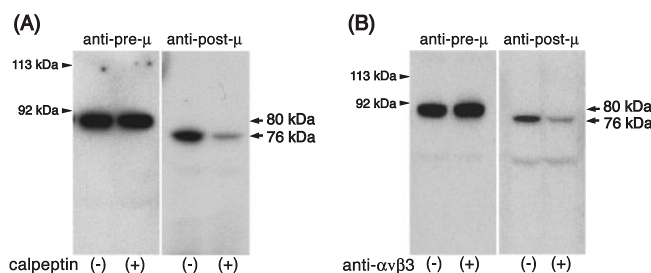
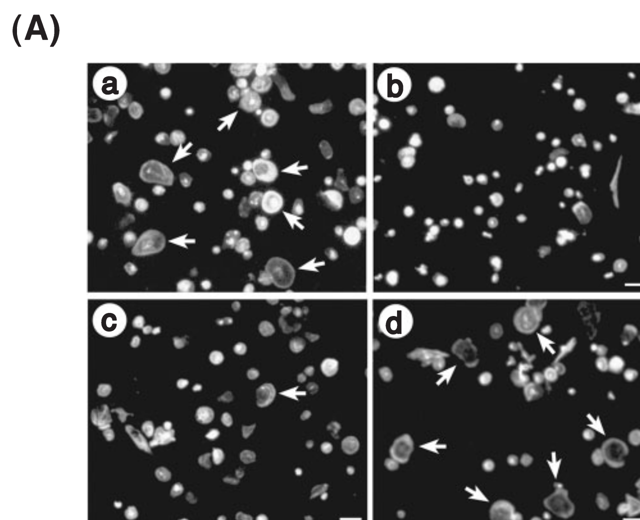


Fig. 4. **Inhibition of μ-calpain activation in osteoclasts upon attachment to bone by treatment with calpeptin and anti-integrin αvβ3 antibody.** Osteoclasts were preincubated with calpeptin (100 μM) or anti-integrin αvβ3 antibody (25 μg/ml) at 37°C for 10 min, then cultured on bone slices in the presence of calpeptin (A) or anti-integrin αvβ3 antibody (B) for 2 h, lysed in the sample buffer for SDS-PAGE, and used for Western blotting. μ-Calpain antigens were detected with anti–pre-μ-calpain (80 kDa) antibody or anti–post-μ-calpain (76 kDa) antibody.

Effects of Various Calpain Inhibitors on Osteoclast Resorption Activity—To explore whether calpain is involved in osteoclastic bone resorption, we first examined the effects of various calpain inhibitors on the resorption activity of osteoclasts. Resorption activity was estimated by the formation of resorption lacunae on a hydroxyapatite-coated disc (osteological disc). Typical examples are shown in Fig. 2A. Membrane-permeable calpain inhibitors such as calpeptin and E-64d inhibited the resorption activity, but an impermeable inhibitor, E-64c, did not (Fig. 2B). These results suggest the involvement of calpain in resorption. Nevertheless, it is known that these peptidyl calpain inhibitors inhibit other cysteine proteases, including cathepsins (*33*). In this study, however, the resorption activity was measured on osteological discs that did not contain organic materials and, thus, it seems very improbable that protease secreted from osteoclasts participates in the resorption process. On the other hand, Schoenwaelder and Burridge reported that calpeptin inhibits the protein-tyrosine phosphatase upstream of Rho, a small GTPase that regulates cytoskeletal reorganization (*34*), although Bialkowska *et al.* (*35*) disagree with this conclusion. Therefore, we also checked the effect



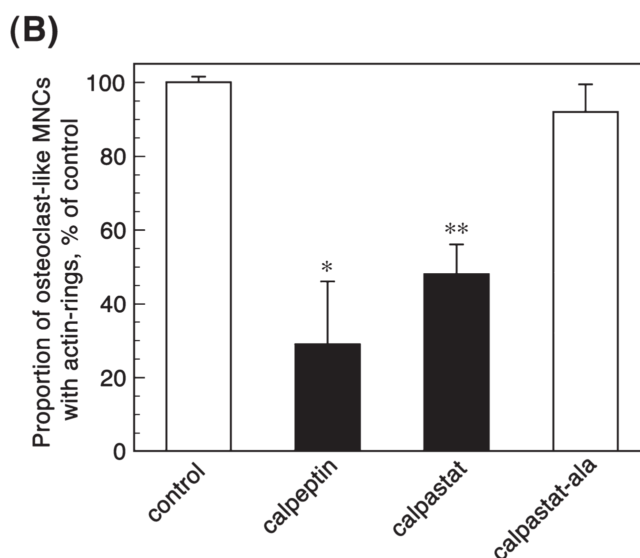


Fig. 5. **Inhibition of actin-ring formation in osteoclasts by treatment with calpain inhibitors.** (A) Osteoclasts were cultured on plastic dishes in the presence or absence of calpain inhibitors for 2 days, and F-actin was stained with a rhodamine-conjugated phalloidin solution. a: Untreated cells (control). b: Cells treated with 100 μM calpeptin. c: Cells treated with 50 μM calpastat. d: Cells treated with 50 μM calpastat-ala. White arrows show typical osteoclasts having actin-rings. (B) The proportion of osteoclasts having actin-rings among total osteoclasts was scored. The proportion in the absence of calpain inhibitors was taken as 100% and the results are expressed as the means ± SE of triplicate cultures. More than 100 osteoclasts were evaluated. Significantly different from control at $^{*}p < 0.05$, $^{**}p < 0.01$.

of calpastat, a cell permeable, calpain-specific inhibitor (see "MATERIALS AND METHODS"). The IC_{50} of calpastat for the inhibition of μ-calpain–mediated caseinolysis *in vitro* is 400 nM. On the other hand, calpastat-ala, a mutant peptide in which alanine replaces conserved calpastatin residues, lacks μ-calpain inhibitory activity (data not shown). These characteristics of calpastat and its derivative are in accord with the previous results of Croce *et al.* (*20*). The results showed the resorption activity to be sig-

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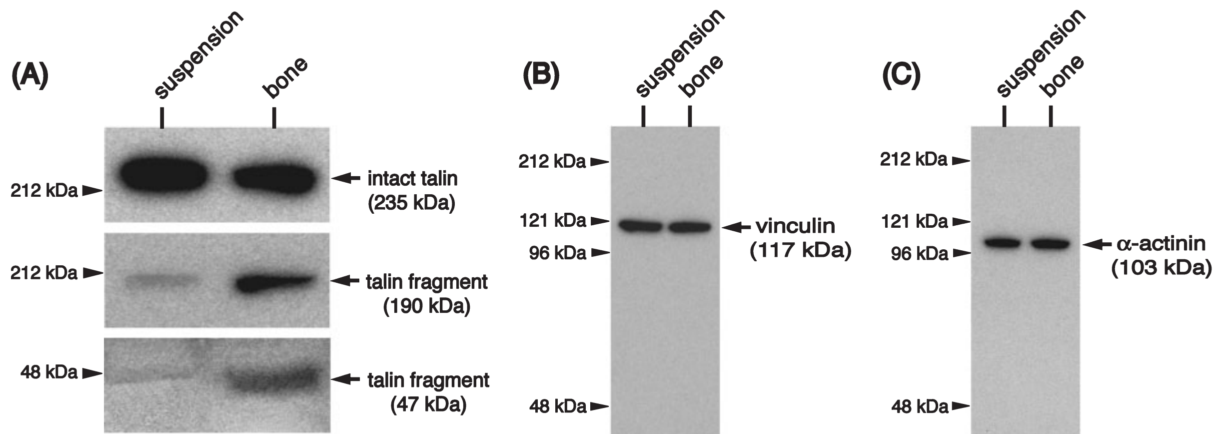

Fig. 6. **Limited proteolysis of talin in osteoclasts upon attachment to bone.** The same number of osteoclasts were kept in suspension or allowed to attach to bone for 2 h, then lysed in the sample buffer for SDS-PAGE and used for Western blotting. (A) Immunostained with anti–intact talin antibody (top), anti–190-kDa fragment (middle), and anti–47-kDa fragment antibodies (bottom) exclusively specific to the calpain-generated fragments of talin. (B) Immunostained with anti-vinculin antibody. (C) Immunostained with anti–α-actinin antibody.

nificantly suppressed by calpastat, but not by calpastat-ala (Fig. 2B). We could not examine the effect of calpastat at concentrations higher than 50 μM because of its cytotoxicity.

Autolytic Activation of μ-Calpain in Osteoclasts upon Attachment to Bone—We next examined the behavior of μ-calpain in osteoclasts upon attachment of the cells to bone, since inhibitor studies suggested the involvement of calpain in osteoclastic bone resorption. Cells in suspension or seeded on bone slices were lysed with Triton X-100–containing buffer, and μ-calpain in the Triton X-100–soluble (TSF) cytosolic and insoluble (TIF) cytoskeletal fractions was analyzed by Western blotting. In suspension, μ-calpain exists exclusively in the soluble cytosolic fraction as an intact 80-kDa form. Upon attachment to bone, however, a portion of the μ-calpain translocates from the cytosolic to the cytoskeletal fraction and is converted from the intact 80-kDa form to the autolyzed (active) 76-kDa form (Fig. 3A and 3B). Autolysed 76-kDa μ-calpain is detected not only in the cytoskeletal fraction but also in the cytosolic fraction (Fig. 3B). The 76-kDa μ-calpain in the cytosolic fraction might be released from the cytoskeletal fraction, because we have previously found that the 76-kDa μ-calpain transfers from the Triton X-100–insoluble to the soluble fraction during platelet aggregation triggered by the binding of an adhesive ligand to integrin αIIbβ3 (*7*).

Inhibition of μ-Calpain Activation by Membrane-Permeable Calpain Inhibitor and Anti–Integrin αvβ3 Antibody—Autolytic activation of μ-calpain in osteoclasts upon attachment to bone is strongly suppressed by treatment with calpeptin, a membrane-permeable calpain inhibitor, which acts to prevent resorption activity (Fig. 4A). On the other hand, a membrane-impermeable calpain inhibitor, E-64c, has no inhibitory effect (data not shown). The activation of μ-calpain is also inhibited by treatment with an antibody against integrin αvβ3, which is responsible for the attachment of osteoclasts to bone (Fig. 4B). This result suggests that μ-calpain activation in osteoclasts is triggered through the binding of integrin αvβ3 on the cell surface to an adhesive ligand on the bone surface.

Inhibition of Actin-Ring Formation in Osteoclasts by Calpain Inhibitors—Osteoclasts change in shape and size depending on the organization of cytoskeletal structures, including actin-ring structures, during the bone resorption cycle. It is considered that actin-ring formation is essential for bone resorption activity. On the other hand, calpain is involved in cytoskeletal reorganization (*8–11*). Therefore, it is of interest to examine whether calpain is involved in actin-ring formation. To assess this possibility, we examined the inhibitory effects of membrane-permeable calpain inhibitors on actin-ring formation. As shown in Fig. 5, actin-ring formation is significantly suppressed by calpeptin and calpastat, but not by calpastat-ala, which lacks calpain inhibitory activity. These results indicate that calpain may contribute to actin-ring formation. To exclude any cytotoxic effect of these inhibitors, we tested the cell viability. The viability of osteoclasts treated with calpeptin (100 μM) or calpastat (50 μM), evaluated using the Live/Dead viability/cytotoxicity kit, was almost the same as that of control cells (data not shown). Therefore, the inhibitory effects of these compounds on actin-ring formation and bone resorption (Fig. 2) were concluded not to be due to their cytotoxicity.

Limited Proteolysis of Talin in Osteoclasts upon Attachment to Bone—Actin-ring structures consist of an actin filament core surrounded by actin-binding proteins such as talin, α-actinin, and vinculin, which link matrix-recognizing integrins to the cytoskeleton (*36*). Interestingly, all these actin-binding proteins are known to be cleaved by calpain (*7*, *37*, *38*). We, therefore, examined whether these proteins are cleaved by calpain upon attachment of osteoclasts to bone. The calpain-mediated cleavage of talin was estimated by Western blotting using antibodies exclusively specific to the calpain-generated C-terminal (190 kDa) and N-terminal fragments (47 kDa) of talin (*6*, *7*). Upon cell attachment, the amount of intact talin (235 kDa) decreased, and the calpain-generated 190-kDa and 47-kDa talin fragments appeared (Fig. 6A). On the other hand, neither a significant decrease in the amount of intact vinculin and α-actinin nor the appearance of any of

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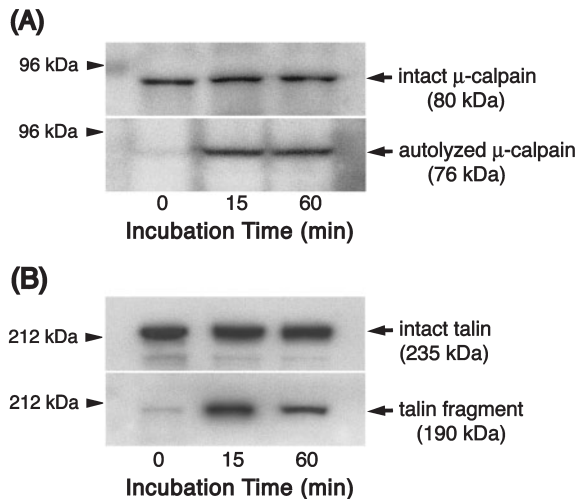

Fig. 7. **Time course of μ-calpain activation and limited proteolysis of talin in osteoclasts upon attachment to bone.** The same number of osteoclasts were allowed to attach to bone for various periods, then lysed in the sample buffer for SDS-PAGE and subjected to Western blotting. (A) Immunostained with anti–pre-μ-calpain (80 kDa) antibody (upper) or anti–post-μ-calpain (76 kDa) antibody (lower). (B) Immunostained with anti–intact-talin antibody (upper) or anti–190-kDa fragment antibody (lower) exclusively specific to the calpain-generated fragment of talin.

their degradation products could be observed upon attachment (Fig. 6, B and C). These results indicate that calpain in osteoclasts selectively cleaves talin upon attachment to bone. It is known that bone resorption activity of osteoclasts appears about 2 h after plating on dentine slices (*39*, *40*), while the autolyzed 76-kDa μ-calpain (active form) and calpain-generated fragments of talin were detected as early as 15 min (Fig. 7). Therefore, the limited proteolysis of talin by calpain in osteoclasts is an early event in the bone resorption process.

DISCUSSION

The present study has shown that membrane-permeable calpain inhibitors suppress the bone resorption activity of human osteoclasts (Fig. 2). μ-Calpain in osteoclasts translocates into the cytoskeletal fraction and is subsequently activated upon attachment of the cells to bone (Fig. 3). In addition, membrane-permeable calpain inhibitors inhibit μ-calpain activation and the actin-ring formation that is essential for bone resorption activity (Figs. 4, and 5). Finally, activated μ-calpain in osteoclasts selectively cleaves talin, a protein associated with actin-ring structures (Fig. 6). From these results, we believe that calpain participates in osteoclastic bone resorption. As far as we know, this is the first report describing the involvement of calpain in osteoclastic bone resorption.

As described above, μ-calpain translocates into the cytoskeletal fraction and is subsequently activated upon the attachment of osteoclasts to bone, as has been observed in platelets upon activation (*5*–*7*, *41*). In platelets, it has been proposed that the translocation and activation of μ-calpain are triggered by the binding of an adhesive ligand, fibrinogen, to integrin αIIbβ3. Osteoclastic bone resorption also requires cell–matrix contact, an event mediated by integrin αvβ3. Therefore, it can be expected that μ-calpain in osteoclasts behaves in a similar manner. Namely, the binding of a ligand on the bone surface to integrin αvβ3 on the cell surface triggers the translocation and activation of μ-calpain. This hypothesis is supported by the fact that the activation of μ-calpain is inhibited by treatment of osteoclasts with a monoclonal antibody against integrin αvβ3 (Fig. 4B). On the other hand, it is also known that signaling molecules, phosphatidylinositol 3-kinase (PI3-kinase), $pp60^{c\text{-}src}$, and rho A, which are demonstrated to be essential for osteoclastic bone resorption, translocate into the cytoskeletal fraction upon the attachment of osteoclasts to bone (*42*). Moreover, PI3-kinase has been shown to be activated in osteoclasts by osteopontin, a bone matrix protein that binds to integrin αvβ3 (*43*). From these facts, it is tempting to speculate that calpain plays a role in cooperation with the above-mentioned signaling molecules in the integrin-mediated signaling pathway during bone resorption.

Integrin αvβ3 is involved in the formation of actin-ring structures that are essential for bone resorption, since osteoclasts from integrin αvβ3-deficient mice do not form actin-rings (*13*). This suggests that calpain participates in actin-ring formation. As expected, membrane-permeable calpain inhibitors significantly suppress the formation of actin-rings (Fig. 5). Therefore, we examined whether actin-binding proteins associated with actin-ring structures are cleaved by calpain. Our results revealed that calpain selectively cleaves talin and generates N-terminal 47-kDa (head domain) and C-terminal 190-kDa (rod domain) fragments upon the attachment of osteoclasts to bone (Fig. 6). Talin links the integrin β subunit to the actin cytoskeleton. The association of talin with integrin β cytoplasmic tails is important in governing integrin activation and clustering (*35*, *44*, *45*). The integrin β tail-binding site of talin exists within the 47-kDa fragment and regulates integrin activation (*7*, *44*–*46*). Interestingly, Yan *et al.* (*47*) reported that the calpain-generated 47-kDa fragment has a 6-fold higher binding affinity than intact talin for the integrin β3-tail. These facts suggest that the generation of the 47-kDa fragment by calpain is necessary for the formation of actin-rings. The 47-kDa fragment may contribute to the construction of a solid actin-ring structure through the activation and clustering of integrin αvβ3. The function of this fragment may also be regulated by phosphorylation, since this fragment is phosphorylated by protein kinase P (serine/threonine kinase) at a level approximately 20-fold higher than that of intact talin (*48*).

There are interesting reports that the actin cytoskeleton plays an important role in the regulation of osteoclast V-ATPase (*49*–*51*). Holliday *et al.* (*50*) demonstrated that V-ATPase binds actin filaments with high affinity, and they proposed that this interaction is an important process controlling the recruitment of V-ATPase from the cytoplasm to the ruffled membrane. Therefore, the limited proteolysis of talin by calpain might relate to the reorganization of the actin cytoskeleton, which is suitable for the recruitment of V-ATPase to the ruffled membrane. This speculation is consistent with the fact that membrane-permeable calpain inhibitors inhibit the formation of resorption lacuna on hydroxyapatite-coated discs, which is affected by the level of protons (Fig. 2).

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Further investigations are necessary to clarify the relationship between the calpain-mediated proteolysis of talin and bone resorption activity of osteoclasts.

It has been reported that the cytoplasmic domain of integrin β3 is cleaved by calpain in endothelial cells and platelets (*35*, *52*). In this study, we could not confirm the calpain-mediated cleavage of integrin β3 upon the attachment of osteoclasts to bone. However, this does not exclude the possibility that integrin β3 is cleaved by calpain during the osteoclastic bone resorption process. Cleavage of the integrin β3 tail by calpain might be related to the disintegration of actin-ring structures, which is a post-resorptive event, through the reversal of integrin activation and clustering. Studies along this line are currently underway in this laboratory. It is also important to verify the relationship between calpain and other signalling molecules such as $pp60^{c\text{-}src}$, rhoA, and PI3-kinase in osteoclastic bone resorption, because it has been reported that calpain cleaves $pp60^{c\text{-}src}$ and rhoA in platelets and endothelial cells (*53*, *54*) and is involved in the regulation of the integrin αIIbβ3-dependent PI3-kinase signalling pathway in platelets (*55*). This information will lead to a better understanding of the definitive role of calpain in osteoclastic bone resorption.

This study was supported in part by research grants from a Grant-in Aid for Scientific Research (B) from the Japan Society for Promotion of Science. We thank Dr. M.M. Dooley-Ohto for reading the manuscript.

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